

Identification of Protein Interactions Involved in Cellular Signaling

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Protein-protein interactions drive biological processes. They are critical for all intra- and extracellular functions, and the technologies to analyze them are widely applied throughout the various fields of biological sciences. This study takes an in-depth view of some common principles of cellular regulation and provides a detailed account of approaches required to comprehensively map signaling protein-protein interactions in any particular cellular system or condition. We provide a critical review of the benefits and disadvantages of the yeast two-hybrid method and affinity purification coupled with mass spectrometric procedures for identification of signaling protein-protein interactions. In particular, we emphasize the quantitative and qualitative differences between tandem affinity and one-step purification (such as FLAG and Strep tag) methods. Although applicable to all types of interaction studies, a special section is devoted in this review to aspects that should be considered when attempting to identify signaling protein interactions that often are transient and weak by nature. Finally, we discuss shotgun and quantitative information that can be gleaned by MS-coupled methods for analysis of multiprotein complexes. *Molecular & Cellular Proteomics* 12: 10.1074/mcp.R113.027771, 1752–1763, 2013.

Virtually every cellular function requires physical protein-protein interactions (PPIs)¹ between cellular proteins. Moreover, cellular functions are critically dependent on the correct assembly of proteins to become functional multiprotein complexes, where there is dynamic interchange of complex components in response to signals, from internal molecular cellular demands, or a cell environment. Well known examples of such multiprotein complexes are RNA splicing, transcription, and translation machineries. In the case of ribosomes and spliceosomes, these complexes reach the molecular weight

of several megadaltons, and they consist of 100–300 different proteins structurally bound with structural and regulatory RNAs (1). However, whereas riboprotein complexes may represent the extreme example of the complexity of protein complexes, proteins involved in cellular signaling have also been described to function as a part of megadalton protein complexes consisting of dozens of different proteins (2). Moreover, the correct functioning of signaling pathways, transmitting signals from cell surface receptors via kinase networks to the nucleus, requires multiple sequential and transient interactions between upstream and downstream components of the particular pathway. This exemplifies the importance of dynamic association and dissociation of proteins in the regulation of cellular response to external and internal cues (Fig. 1). As such, the signaling relays of every docking interaction between proteins can be considered as a mode of regulating protein function, and these interaction surfaces also are subject to regulation by post-translational protein modifications or, for example, mutations.

Recent studies have emphasized that PPIs define the specificity in signal transduction (4). Based on this, an hypothesis could be drawn that PPIs with signaling proteins differ in the physiological and pathological situations. Accordingly, characterization of protein interactions with signaling proteins could be used to elucidate the mechanistic basis of pathogenesis in different diseases (5). In addition, this type of analysis might form a basis for the design of specific therapeutic tools to inhibit interactions that specifically support pathological behavior of the cell. This could be achieved by generating specific cell-permeable peptides or by small molecules (6, 7). Whether PPI between specific proteins can be subjected to inhibition by small molecules or peptides depends on the structure of the protein domains mediating the interactions, but in principal every protein interaction depicted in the hypothetical signal transduction pathway in Fig. 1 could be considered as a potential target for therapeutic intervention. The most encouraging examples of therapeutic use of blockage of signaling PPIs is the peptide inhibitor of the JNK-JIP1 interaction and small molecule inhibitors of p53-MDM2 interaction and Bcl-2 complexes (6–9). These inhibitors are currently in clinical development in the application areas of hearing loss and cancer, respectively. In addition, recently, the oncogenic Bcr-Abl kinase has been targeted with an engineered monoclonal antibody that disrupts the intramolecular interaction between the Src homology 2 and kinase domains

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¹ The abbreviations used are: PPI, protein-protein interaction; AP-MS, affinity purification coupled with mass spectrometry; TAP, tandem affinity purification; Y2H, yeast two-hybrid; PTM, post-translational modification; SILAC, stable isotope labeling by amino acids in cell culture; EGFR, EGFR receptor.

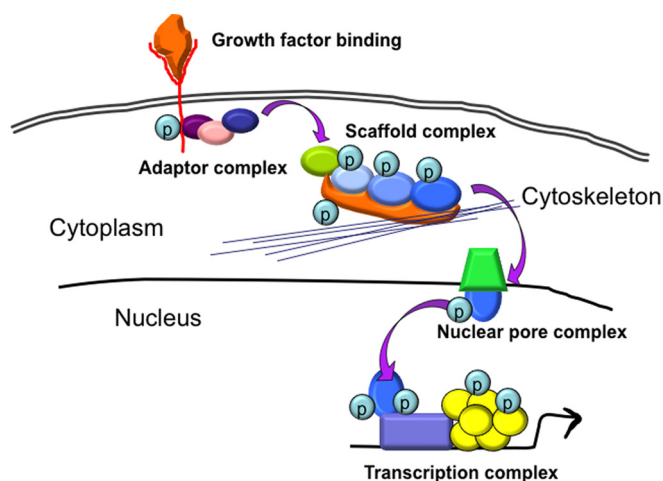


FIG. 1. **Protein interactions mediate cellular responses.** A hypothetical signal transduction pathway where sequential and transient interactions between upstream and downstream components of this pathway are active. All depicted interactions both between signaling proteins and with other cellular constituents are critical for proper signaling. They also are potential targets for disease causing alterations as well as for therapeutic targeting.

in Bcr-Abl resulting in kinase inhibition, and it completely abolished leukemia formation in mice (10).

Mechanisms and Regulation of Signaling Protein Interactions

PPIs are most often mediated by distinct PPI domains in the proteins, but many interactions also occur outside of the classical PPI domains and/or they involve unfolded regions on the proteins. About 80 different PPI domains have been identified, and many proteins consist of several PPI domains. PPI domains may also form functional units, such as the RING domain that is both a PPI domain but also functions as a ubiquitin ligase by simultaneously binding to ubiquitination enzymes and their substrates (11). PPI domains, like other protein domains, are determined both by the amino acid sequence and by the three-dimensional structure of the domain, due to the intermolecular interactions of the proteins. The definition of a protein domain includes that it is self-stable, *i.e.* that a domain forms a functional fold, even if it is fused to another protein. This property is now used extensively to create fusion proteins to study the role of PPIs and how they determine the signaling specificity, and even to create artificial proteins that have been shown to re-direct signaling networks due to acquiring new PPIs (12).

PPIs can be classified according to the nature of chemical bonds between the interacting proteins. By doing this, we can consider covalent and noncovalent bindings. The highest affinity association between two proteins is mediated by a covalent bond that is formed either by the sharing of outer atomic orbital electrons of the amino acids forming the contact between two proteins or by disulfide bonds. Covalent PPIs are generally rare, except for covalent attachment of

ubiquitin or SUMO proteins to signaling proteins. However, the most common types of PPIs are based on noncovalent bonds, which allow transient association and dissociation of proteins, a critical property for dynamic regulation of cell signaling. Noncovalent interactions are based on hydrogen bonds, ionic interactions, van der Waals interactions, or hydrophobic bonds between the interacting proteins. Noncovalent interactions are generally rather weak, but in most of the cases, the two interacting proteins form simultaneously a number of noncovalent bonds between amino acids of the interaction domain, and the actual affinity between two proteins is a combination of the affinities of these multiple weak bonds. Understanding the nature of PPI bonds is important, because the chemical nature of the various bonds is differentially sensitive to changes in the cellular environment, as well as ion composition, pH values, and other chemical characteristics. These aspects are particularly important when studying transient and weak interactions between signaling proteins.

Many cellular signaling proteins are enzymatically active and have the capacity to post-translationally modify their substrates (13). The most common and best understood post-translational modification (PTM) involved in the regulation of PPIs is protein phosphorylation. Many other PTMs, such as protein acetylation or hydroxylation, have also been shown to potentially regulate signaling protein interactions (13). PTMs may affect the interaction between two proteins principally by two mechanisms. First, PTMs may change the chemical properties of the modified amino acid thereby potentially creating or abolishing a possibility to create chemical bonds between PPI domains. Second, PTMs are known to affect protein folding and secondary structure of PPI domains. Thereby, the PTM may either expose a new PPI domain in the surface of the protein or lock a PPI domain to be inaccessible for binding. Well known examples of proteins that specifically bind to PTM sites are 14-3-3 proteins that recognize phosphorylated amino acids of a particular sequence (14) and the interaction between transcription factor HIF-1 α and ubiquitin ligase VHL, which is dependent on hydroxylation of HIF-1 α by specific hydroxylase enzymes (15).

Often the activation of signaling cascades also involves multiprotein complexes that contain both upstream and downstream effectors of the given pathway. For example, initiation of growth factor signaling by growth factor receptors requires the interaction of the intracellular receptor tail with adapter proteins Grb2 and Sos, which in turn interacts with, and activates, Ras GTPases resulting in the recruitment of Raf proteins to the protein complex in the vicinity of the plasma membrane (Fig. 1). In addition to adapter proteins linking two or more signaling proteins via PPIs, the components of signaling pathways are in some cases tethered together by structural scaffold proteins that provide specific binding sites for each component of the pathway (Fig. 1) (16). Whereas structural scaffolds establish multiprotein complexes, scaffold proteins are also considered to promote the transmission of

TABLE I
Potential limitations and solutions in the basic yeast two-hybrid screening

| Limitation or problem in Y2H analyses | Possible solution |
|---|--|
| Bait protein is toxic/growth limiting to yeast | Test a smaller/different fragment of the bait protein |
| Bait protein activates reporter genes alone (auto-activation) | Test a smaller fragment of the bait protein or use the split-ubiquitin Y2H system |
| Protein-protein interactions involving the N terminus of the bait are blocked due to the N-terminal transcription factor fusion | Choose a Y2H system with a reverted polarity (27) |
| Screening for Tyr(P)-dependent interactions | Use a yeast strain expressing a tyrosine kinase (28) |
| Investigation of a ternary protein complex | Use a Y2H system that allows for expression of a third soluble none-fusion protein together with the two transcription factor fusion proteins (three-hybrid) |
| The bait protein is a membrane integral protein and cannot translocate to the nucleus | Use a Y2H variant like the split-ubiquitin two-hybrid (29) |

cell signals by ensuring sufficient spatial concentration of pathway components and thereby facilitating phosphorylation reactions between interacting kinases (Fig. 1). Importantly, scaffolds themselves are also subject to PTM-mediated regulation of their function. One example of such is ERK-mediated phosphorylation of the scaffold protein IQGAP, which in turn results in increased interaction of IQGAP with the upstream activator of ERK, MEK (17). This phosphorylation-sensitive protein interaction mechanism thereby potentially creates a positive feedback loop promoting ERK pathway activity. Cytoskeletal proteins are often considered as contaminants in protein interaction identification studies. However, it is becoming increasingly clear that cytoskeletal proteins have a very important function in providing structural support for protein complexes and for signaling protein trafficking, in addition to their roles in the cytoplasm and the nucleus (18). Some of the published examples of a functional relevance of cytoskeleton interactions for MAPK signaling include stress-regulated interaction between Raf-1 and keratin 8 (19), as well as MEKK3 scaffold (osmosensing scaffold for MEKK3) binding to actin, which was shown to be required for sorbitol-induced p38 activation (20).

Identification of Signaling Protein Interactions

The current literature that covers signaling protein interactions is overwhelming with thousands of published examples. Therefore, rather than reviewing this extensive literature of different signaling protein interactions, we will describe a selection of methodological approaches used to successfully identify and characterize signaling protein interactions, with the aim to provide a basic framework of operation for the molecular interrogation of such PPIs.

Two-hybrid Techniques for Identification of Signaling Protein Interactions

The yeast two-hybrid (Y2H) technique for the detection of PPIs was first described in 1989 (21). In the subsequent 2 decades, this technique has flourished and provided a huge

amount of information on signaling protein interaction networks (22). Today, the traditional yeast two-hybrid technique remains widely used, and its accessibility has increased due to many commercial service providers and core facilities offering Y2H screening services. The basis of the technique is that two functional domains of a transcription factor can be physically separated into individual proteins that, when brought into close proximity by interacting proteins, each fused to one of the transcription factor domains, reconstituting the transcription factor's ability to regulate gene expression (23). The technique exploits the flexibility of yeast transcription factors like Gal4 and LexA to carry out their functions as separated domains fused to almost any other protein. The protein of interest (bait) is cloned as a fusion protein with the DNA binding domain of a yeast transcription factor. A library of cDNA fragments (prey) derived from tissues or a cell line of interest (such as brain, testis, or HeLa cells) was cloned as fusion proteins with the other half (activation domain) of the transcription factor. These two domains of the split transcription factor are unable to interact with each other or activate gene expression alone. However, if the cDNA fragment fused with the transcription factor domain is able to interact with the bait protein, the two halves of the transcription factor are brought into close proximity thus reconstituting a functional transcription factor. This is able to activate transcription of specific reporter genes that are used as the basis of the screening. Depending on the system and the yeast strain used, the reporter genes vary. Today, there are several variants of the technique with different reporter genes, but the same basic principles apply to all Y2H-screening systems. Y2H screening has been typically used to identify PPIs related to a specific protein of interest. However, Y2H screening is also applicable for high throughput screening of interactions (23). However, as with every technique there are several limitations in basic yeast two-hybrid screening. These are summarized in Table I. A particularly important limitation regarding signaling protein interaction studies is that protein interactions based on tyrosine phosphorylation cannot be screened in a

normal yeast host strain, because yeast cells do not express tyrosine kinases. Some investigators have attempted to overcome this restriction by screening in a yeast strain expressing a tyrosine kinase ectopically (24). Another limitation is that the proteins that are used as bait cannot exhibit transactivation activity in the used reporter system. This limitation rules out (for example) many proteins involved in transcriptional regulation of gene expression. A third major limitation, which is particularly important to consider when working with cytoplasmic signaling proteins, is that in the basic Y2H system the PPI needs to take place in the nucleus in order for the reconstituted transcription factors to bind to the reporter gene promoters. For further explanation of the technique and history of the yeast two-hybrid, we refer the reader to an excellent review by Fields (25).

To overcome Y2H limitations, investigators interested in protein interaction occurring at the membrane have invented variations of the classical Y2H system (22). One such approach is the split-ubiquitin system that has been further developed into approaches compatible with screening for PPIs from random protein libraries (26, 27) and for detection of altered protein conformations (28). These are also based on protein fragment complementation, as the methods described above. Ubiquitin proteins can be separated into two stable halves, N- and C-terminal (N_{ub} and C_{ub}), and when fused to two interacting proteins, these halves are brought together to reconstitute a ubiquitin molecule that can be recognized by cytosolic yeast-deubiquitinating enzymes. These enzymes cleave ubiquitin from proteins. When an integral membrane bait protein is fused to C_{ub} linked with an artificial transcription factor, this factor is excluded from the nucleus. However, when the bait interacts with a prey fused to N_{ub} resulting in the reconstitution of ubiquitin, cleavage of the transcription factor occurs, and it is released into the nucleus to activate transcription of a reporter gene. In yet another system, the deubiquitinating enzyme produces a rapidly degraded form of a reporter protein, Ura3p, that switches yeast from uracil-independent and 5-fluoro-orotic acid-sensitive to become uracil-dependent and 5-fluoro-orotic acid-insensitive. The details of these systems and their use is described in detail in Ref. 29.

As with all screening systems, the Y2H techniques described above can lead to identification of false positive interactions, and thus all interactions identified using Y2H need to be validated with additional methods such as immunoprecipitation or pulldowns. Alternatively, mammalian two-hybrid methods can be used to validate Y2H-based interactions, and these have been applied for large interactome datasets from different species (30–33). Because many critical co-factors and modifications are often absent from yeast, the ability to screen for interactions in mammalian cells allows for interactions to be analyzed in a more native cellular context for most biomedical areas. There are many different variants of the mammalian two-hybrid systems. The most basic one is conceptually the equivalent of the Y2H system where the inter-

action of two proteins fused to the yeast Gal4 DNA binding domain, and the herpes simplex virus VP16 protein (a transcriptional activator in mammalian cells) results in the activation of a reporter gene (like luciferase), thus allowing the monitoring of protein interactions. A highly successful example exploiting the luciferase-based mammalian two-hybrid method (34) is the high throughput comprehensive identification of tissue-specific physical interactions of transcription factors, and it revealed that combinations of transcription factors are critical for determining cell fate (35). Several other methods have been described as well, which are all based on some version of the protein complementation concept; enzymes or fluorescent proteins are split into two domains, and when a PPI takes place between the fused proteins to these domains they reconstitute the enzyme activity (like β -galactosidase) or fluorescence (like yellow fluorescent protein). Variations of the published techniques are summarized in Ref. 36. Such assays involving bimolecular fluorescence complementation are being increasingly used to detect PPIs especially in the context of living cells (37, 38). In addition, the system could be applicable for high throughput assays if coupled with FACS-based sorting of fluorescent cells (39). However, other methods detecting PPIs, like the combination of fluorescence resonance energy transfer and fluorescence lifetime imaging, which results in a powerful tool for the exploration of PPIs between two fluorescently tagged proteins, can provide better sensitivity especially in the case of investigating specific cellular interactions. As the yeast systems allow for screening with unsurpassed throughput and coverage, it is likely that mammalian two-hybrid method techniques will be more useful for validation of putative interactions and more focused studies of the dynamics of the signaling protein interactions.

Affinity Purification Methods for Identification of Signaling Protein Interactions

As stated above, virtually every cellular function is critically dependent on the assembly of proteins to multiprotein complexes. Therefore, even though Y2H and other earlier methods have revolutionized the studies of PPIs, there has been an obvious need for developing methodology to characterize and discover the components of cellular multiprotein complexes. Identification of multiprotein complexes from living cells is generally based on two technical steps where the first is affinity purification of the protein of interest (bait) and the proteins physically associated with the bait protein, and the second step is mass spectrometry (MS)-based protein identification of the affinity-purified material, referred to as AP-MS (affinity purification coupled with mass spectrometry) from hereon (40–42). In addition to using an epitope-tagged protein of interest as the bait, an alternative application of AP-MS is the identification of target proteins for small molecule drugs immobilized in affinity columns. These chemical biology ap-

proaches, although very relevant to the understanding of cellular signaling, have been reviewed elsewhere (43–45) and will thus not be discussed further in this review.

Interestingly, based on comparative quality assessment report by Vidal and co-workers (23), comparing high throughput protein interaction data derived either from Y2H experiments (31, 33) or by AP-MS (46, 47), it was concluded that although data derived by both approaches are of equally high quality, the nature of protein interaction networks derived from these experiments is fundamentally different and complementary in nature (23). These conclusions further emphasize that the choice of the right methodology (*i.e.* two-hybrid *versus* AP-MS) is critical and should be carefully considered in relation to the scientific question to be addressed.

One of the obvious benefits of AP-MS for studying cellular signaling is that they allow identification of context-dependent PPIs (42). This includes isolation of protein complexes from different cellular compartments and organelles such as cytoplasm, nucleus, nucleoli, or mitochondria. When applied in a comparative experiment, PPI data enable the comparison of (for example) different cellular signaling states. With regard to studying mechanisms of cell signaling, context-dependent variables may be of great importance for understanding the dynamic mechanisms by which activity and function of cellular signaling proteins are regulated. Blueprint examples of signaling state-dependent interactions are intranuclear shuttling of nucleolar proteins and their interactions with nucleoplasmic transcription factors in response to cellular stress (48–51).

As noted above, most of the signaling interactions are thought to fall into the category of weak and transient interactions. For example, phosphatases and protein kinases have traditionally been very challenging as baits in protein-complex purification attempts. Therefore, it is important to consider, in addition to the PPI identification method, the purification conditions, so that these best support the stability of the endogenous signaling protein complexes. For example, Ca^{2+} -chelating EGTA or EDTA is commonly used in purification buffers to mimic the environment inside living cells. However, some of the PPIs are dependent on divalent cations such as Ca^{2+} and Zn^{2+} , and use of either EDTA or EGTA supposedly would brake these interactions. In fact, one study demonstrated that the addition of EGTA dramatically modified S6K1 signaling protein complex formation (52). Another example of the importance of protein interaction purification conditions is our own attempt to purify a protein complex associated with the transcription factor c-Jun. Ultimately, success here was fully dependent on whether we were capable of retaining the Ser-62-phosphorylated form on c-Jun throughout the entire TAP purification protocol, by using phosphatase inhibitors (51).² Phosphorylation dependence of the interaction between c-Jun and its co-factor topoisomerase I was subsequently verified by independent approaches (53). These are illustrative

examples that, especially when low affinity interactions between transiently interacting signaling proteins is concerned, very careful attention should be given to the experimental conditions used.

TAP Methods for Identification of Signaling Protein Complexes

Whereas affinity purification techniques for purification of proteins and protein complexes had been established earlier (40), there were several obstacles to overcome before affinity purification could be used for systemic analysis of protein interactions from living cells. In particular, it was reasoned that the use of affinity tag-based approaches, rather than antibodies specific for bait proteins, would be necessary to develop purification protocols that would be of generic nature, *i.e.* that the purification conditions would not need to be adjusted specifically for each bait protein. Also, contaminating proteins nonspecifically bound and released from antibody columns were a problem. In addition to the lack of an efficient and selective protein purification methodology, MS-based approaches to identify the components of protein complexes from living cells were not realistic prior to the establishment of better (more sensitive) protein identification workflows and the availability of complete, species-specific, genome and protein sequence databases (40). Currently, quantitative MS is the method of choice for studying dynamic signaling protein interactions and is reviewed later. To address the above explained obvious challenges related to protein complex purification for MS identification, Seraphin and co-workers (54) developed a novel TAP protocol for the purification of large protein complexes from yeast under native conditions. The method was based on tagging proteins with a combination of protein A and calmodulin peptide tags that were linked with a cleavage site for tobacco etch virus protease. They demonstrated that protein complexes purified with TAP technique from yeast cells were very low in contaminating background proteins (54). Because of its generic nature, the TAP technique was shown to be suitable for large scale interactome studies in yeast (46). Following the initial publication of successful experiments with TAP in mammalian cell lines (51, 55), the methodology was also used to map human signaling protein interaction networks (56, 57). Although originally referring to specific combinations of protein A and calmodulin-binding peptide tags developed by Seraphin and co-workers (54), the TAP purification is nowadays used as generic name for techniques that use a combination of two tags and two sequential steps of affinity purification. There are several excellent reviews published that describe in detail the different TAP strategies developed and their applications (41, 42, 58).

Advances in Affinity Purification Methods for Identification of Signaling Protein Complexes

The establishment of so-called TAP techniques for AP-MS was a technical revolution that immensely contributed to our

² J. Westermarck, unpublished observations.

understanding of the complexity of intracellular mechanisms involved in the regulation of cell behavior. However, it was quickly realized that long lasting TAP purifications were not particularly suitable for the identification of transient and weak PPIs between signaling proteins (59). To alleviate this problem, several generic one-step protein complex purification methods, such as the FLAG tag or Strep tag methods, were established and demonstrated to be compatible with MS-based signaling protein complex identification (59–62). In addition to the speed of the purification process, another general advantage of most of the epitope tags used for one-step protein complex purifications is their relatively small size as compared with either TAP epitopes or, for example green fluorescent protein (GFP), that have also been successfully used as an epitope tag for AP-MS (63). For example, FLAG and Strep epitopes contain only eight amino acids, making these epitopes very unlikely to interfere with the structure or function of the bait protein (60, 62). However, it should be mentioned that in many cases these epitopes are used as multimers to increase the affinity to purification matrix. Even as a multimer, the size of such epitopes usually does not exceed that of 20 amino acids.

Many of the one-step epitopes reviewed in Refs. 60, 62 have been successfully used for isolation and MS-based identification of signaling protein complexes (61, 64–67). However, out of these studies, the most robust evidence for the usefulness of one-step purification of signaling protein complexes was recently published by Tyers and co-workers (65). In their paper, they identified a kinase and phosphatase interaction network of 1844 interactions by using either HA-tagged or FLAG epitope-tagged bait proteins expressed in yeast. The bait proteins consisted of 201 active kinases or kinase regulatory subunits and 75 phosphatases or phosphatase regulatory subunits (65). In another more focused example of the successful identification of kinase protein complexes by a one-step affinity purification, Siu and co-workers (64) recently identified protein complexes associated with three isoforms of p38 mitogen-activated protein kinase proteins by using FLAG epitope-tagged proteins expressed in *Drosophila* S2 cells. In this work, they identified altogether 46 interaction partners for three studied p38 isoforms and demonstrated surprisingly low redundancy between interactomes of these p38 proteins (64).

In addition to FLAG- and HA epitope-based AP-MS, non-antibody-based approaches such as the Strep tag provide an interesting alternative due to nondenaturing competitive elution of the Strep-tagged bait protein and the associated protein complex (61, 68). The Strep tag peptide binds to an engineered Strep-Tactin affinity matrix with an affinity that is comparable with the very high affinity interaction between biotin and streptavidin. However, unlike the covalent interaction between biotin and streptavidin, the interaction between the Strep tag peptide and Strep-Tactin can be (very) efficiently eluted by simply adding competitive desthiobiotin to the binding buffer (61, 68). This feature enables combination of very

high recovery of the bait protein (usually around 80–90% in the final eluate) and fast and efficient elution of the intact protein complex (61, 68). In addition, when compared with antibody-based methods, antibody contaminations in the final eluates subjected to MS are obviously not a concern. Another useful feature of the Strep tag-Strep-Tactin interaction is its resistance to a large number of chemicals and varying salt concentrations (61, 68). This allows more choices for the composition of the buffer reagents and sample preparation method. Published evidence supporting the usefulness of the Strep tag for AP-MS-based identification of novel signaling protein complexes includes protein complexes associated with PP2A, IKAP, and transcription factor Nrf2 (61, 66, 67). More recently we have identified a novel PME-1 phosphatase inhibitor protein-associated protein complex with the one-step Strep tag method.³ MS analysis of co-purified proteins demonstrated the existence of target phosphatase PP2A as well as several PP2A target kinases, further supporting the usefulness of this approach for identification of transient phosphorylation-dependent interactions. Interestingly, competitive elution of the Strep tag-resin interaction by desthiobiotin in nondenaturing conditions (68) facilitates enzymatic activity measurements from the final eluates, as was originally demonstrated from the PP2A complex (61). This might be of particular interest when studying enzymatically active signaling proteins such as kinases, phosphatases, acetylases, or methylases, and it might allow for the establishment of functional rather than MS-based screening of the protein complexes.

Increased probability of the identification of low affinity and transient protein interactions by using one-step methods does not come without a price; it is clear that fewer purification steps also result in the increased risk of identifying contaminating proteins that are not true interactors of the bait but are bound nonspecifically to the beads and eluted together with the bait protein and true interacting proteins. One potential approach to address this problem and to distinguish between nonspecific and true interacting proteins is characterization of common contaminants binding to employed affinity matrix (bead proteome, “beadome”). By using this strategy, Lamond and co-workers (63) were able to use one-step purification method for reliable MS identification of protein complexes from mammalian cells. Another successful example of using the beadome approach to discover the actual interactome for signaling protein followed by one-step AP-MS was recently published by Siu and co-workers (64). In addition to simple threshold models comparing bait-specific and beadome peptides, recent studies have introduced more sophisticated computational approaches to calculate the probability of a true interaction. These methods include a statistical model called (SAINT) (69, 70). SAINT assigns the number of

³ Y. R. Pokharel, G. L. Corthals, and J. Westermarck, unpublished results.

peptide identifications for each interactor to a probability distribution, which is then used to estimate the likelihood of a true interaction. SAINT has been demonstrated to facilitate identification of high confidence interactomes of both yeast and human kinases and phosphatases (65, 71). Other approaches for computational analysis of nonspecific interactions are reviewed by Gavin *et al.* (59).

An alternative approach to deal with the delicate balance between yield and purity of AP-MS experiments is to use cross-linking agents that stabilize the PPIs between bait and the associated proteins. Several different cross-linking agents and protocols have been shown to be compatible with subsequent MS-based peptide analysis (42, 72, 73). In addition to cross-linking approaches, where the cross-linking agent is added to the cultured cells to stabilize the interactions before affinity purification (72, 74), cross-linking of already purified complexes has recently been shown to facilitate MS-based identification of PPI interfaces between protein complex components (42, 75). This approach may significantly accelerate development of therapeutic PPI inhibitors (6, 7) as well as provide important insights toward understanding the mechanistic basis for regulation of protein interactions. Moreover, several recent studies have shown usefulness of this approach for structural modeling of protein complexes (73, 75).

In addition to MS-based identification of novel protein interactions, affinity purification methods can be employed for high throughput mapping of protein interaction networks, by using expression libraries coding for fluorescently labeled proteins. The luminescence-based mammalian interactome mapping (LUMIER) assay that was originally developed by Wrana and co-workers (76) is a good example. Here, the FLAG-tagged bait proteins and *Renilla* luciferase-tagged prey proteins are co-transfected to mammalian cells, and the protein interaction is detected by measuring luciferase signal bound to anti-FLAG-coated beads in a 96-well plate format. In this original report, the authors demonstrated the capacity of the methodology to map signaling interaction networks between TGF- β core signaling pathway components and 518 other proteins in an automated fashion (76). Very recently, Lindquist and co-workers (77) reported further development of the LUMIER method and applied it to identify specific protein interactions between Hsp90 and 1874 signaling proteins, including protein kinases, transcription factors, and E3 ubiquitin ligases. In their LUMIER with bait control (LUMIER with BACON) assay, the abundance of the bait is measured after reading the luciferase with ELISA using a different polyclonal antibody against the FLAG tag (77). In this elegant study, they could demonstrate that, when compared with transcription factors, Hsp90 binds preferably to active kinase proteins and that this binding was dependent on both kinase recognition by Hsp90 co-chaperone CDC37 and conformation of the interacting kinase. These studies have convincingly demonstrated the usefulness of LUMIER-type assays for establishing protein networks in mammalian cells. However, as

in each of these studies the generation of the data required thousands of individual binding reactions to be measured, subsequently requiring the establishment of automated robotic liquid handling systems, these methods may not be readily suitable for standard laboratory settings.

Method of Choice for AP-MS Experiments and Other Considerations

What seems to be a general conclusion from comparing the different affinity purification techniques is that although TAP approaches produce less background and are suitable for identifying stable protein core complexes with high fidelity, one-step approaches are indeed better for identification of weak and transient interactions. This notion was originally supported 10 years ago by comparison of two large scale PPI studies from yeast, one using the TAP strategy and another the FLAG-tagged bait proteins isolated by one-step immunoprecipitation (31, 40, 46, 78). Although there are certain limitations in comparing the interaction data between these two studies, it was noted that protein complexes isolated by FLAG affinity column contained more interacting proteins than those obtained by TAP strategy (78). More recently, Gstaiger and co-workers (79) showed that there was clear decrease in the abundance of both bait protein and in the interacting protein complexity in the final eluates after two purification steps as compared with one-step purification by using their elegant SH TAP tag (Strep-HA) purification method. Together, these examples clearly support the notion that due to the risk of losing weak PPIs during the purification protocol, it is advisable to aim for as few purifications steps and short purification time as possible. Based on these considerations and on recent development of powerful computational methods, such as SAINT (69, 70, 71), our conclusion is that a combination of these approaches might provide the best method for reliable identification of low abundance and weak interactions, including signaling protein interactions. However, it should be noted that not all signaling protein complexes are transient. One of the most studied signaling protein complexes by AP-MS methods is PP2A, which presents itself as either a dimeric or trimeric core protein complex (80). Numerous PP2A interaction partners have been recently identified by both TAP as well as FLAG and Strep tag purification methods (57, 61, 75, 79, 81).

It is noteworthy to recall, even though currently used quantitative methods emphasize the importance of repeatability in weighting the likelihood of an identified interaction to be true, our own experience is that even one-time identification of a few peptides of a previously unknown protein may lead to the discovery of an entirely new biological concept, provided the identified interaction and its functional relevance are properly verified by subsequent experimentation (51, 81). This is best exemplified by AP-MS identification of a protein CIP2A as a novel interaction partner for the PP2A complex (81). Although

this protein was originally identified as a putative PP2A binding partner only in one single experiment, and only based on seven identified peptides with relatively low MASCOT scores (81), the functional relevance of this interaction in the regulation of PP2A tumor suppressor activity in human cancer has been thereafter been verified by numerous independent studies (80, 82–86). Finally, when considering the reliability of the identified putative interaction, the functional classification of candidate interactors to “relevant” *versus* “nonrelevant” may not be advisable without further supporting data. Protein interactions of signaling proteins with cytoskeletal proteins are classical examples of such interactions that might be omitted using such classification, notwithstanding the vast literature of relevance of these interactions for regulation of cellular signaling (18–20, 66, 87).

Considerations for AP-MS Experiments

The checklist of issues to consider when planning for AP-MS experiments for identification of signaling protein interactions are as follows. (a) Choice of the affinity purification method: TAP *versus* one-step AP protocols? (b) Biochemistry of the bait protein and expected protein complexes: need for a specific buffer composition for retaining PPIs? (c) Need of and suitability of cross-linking approaches? (d) Need of and suitability of metabolic labeling such as SILAC? (e) Determination of specificity of interactions; “beadome” and computational methods for data filtering? (f) Specific applications: enzymatic activity measurements, protein interaction domain identification by cross-linking, or LUMIER-type assays? SILAC is described below.

Coupling of Quantitative Mass Spectrometry Analyses with Protein Interaction Studies—By the end of the 1990s, mass spectrometry had overcome several technical limitations to establish the technology as the method of choice for fast and facile identification of proteins from gel bands or in solution-digested protein mixtures. Further technological developments over the last 10 years have seen MS evolve to allow simultaneous qualitative and quantitative measurements of protein abundances (88). For characterization of the composition and quantity of PPIs, MS advances would allow the detailed mapping of multiprotein complexes. At the same time, ongoing improvements in the site-specific identification of PTMs have evolved, such as phosphorylation. Here as well, these methods combined with quantitation have ushered in sophisticated experimental approaches integrated with MS (88).

It is perhaps without surprise that in recent years the notion of performing PPI studies using quantitative MS measurements has gained wider appreciation, as there are two considerable shortcomings of PPI data that can be avoided when using quantitation. First, protein complexes and networks are not static entities as structures and compositions can change dynamically in response to cellular signals. Second, the false

positive error rates can be reliably estimated based on quantitative interaction experiments (89). Thus, quantitative MS approaches, along with appropriate control experiments, can identify changes in the compositions of protein complexes and can distinguish background contaminants in protein complex purifications from true interacting partners. An additional important aspect is the already discussed role of PTMs that frequently modulate the composition and location of complexes.

MS-based quantitative methods have existed for a long time as an application of stable isotope labeling, originally and solely used for absolute measurements (88). Current methods that have been developed over the last 10 years are mostly derivatives from these original concepts in that the protein abundance is determined from the area under the curve of eluting peptides, or the summed intensity. The two main methods that are currently used are based on the following: 1) chemical incorporation or “tagging,” where chemical modification of proteins in a site-specific manner is performed using a derivatization reagent; and 2) biological or metabolic incorporation, where labeling of the peptide/protein is achieved by growing cells in media enriched in stable isotope-containing amino acids (SILAC). A further recent development is to use so-called label-free methods, where multiple LC-MS or LC-MS/MS experiments are compared with each other and analyzed for their similarities and differences. In both isotope-based and label-free experiments, quantification is achieved by analyzing either of two sources of information. As developments in computational methods for label-free MS are only now emerging, we will restrict this review to methods that are robust and generally applicable today.

Application of Quantitative MS to PPIs—Several studies have attempted to gain insights into the dynamics of protein complexes in the context of signal transduction by establishing a correlation between the state of the complex components and protein phosphorylation (90, 91). Smolka *et al.* (91) were among the first to show the power of temporal quantitative analyses by studying changes in the composition and phosphorylation of Rad53 in response to DNA damage. Rad53 is an essential checkpoint serine/threonine kinase in the DNA damage-response pathway in *Saccharomyces cerevisiae* and becomes hyper-phosphorylated and activated following DNA damage. Prior to their studies, specific phosphorylation sites of Rad53 had not been mapped nor had the role of several Rad53-associated proteins been clarified in the context of how they might affect DNA damage. Their *N-isotag* specifically labeled the primary amines present at the N terminus and lysine residues of peptides via formation of stable amide bonds, similar to what happens with the iTRAQ method (92), which has now become a popular method for such experiments due to the ability to now incorporate anywhere between 2 and 8 labels in a single experiment. Nevertheless, the *N-isotag* approach by Smolka *et al.* (91) was among the first to highlight that integrated quantitative MS is generally

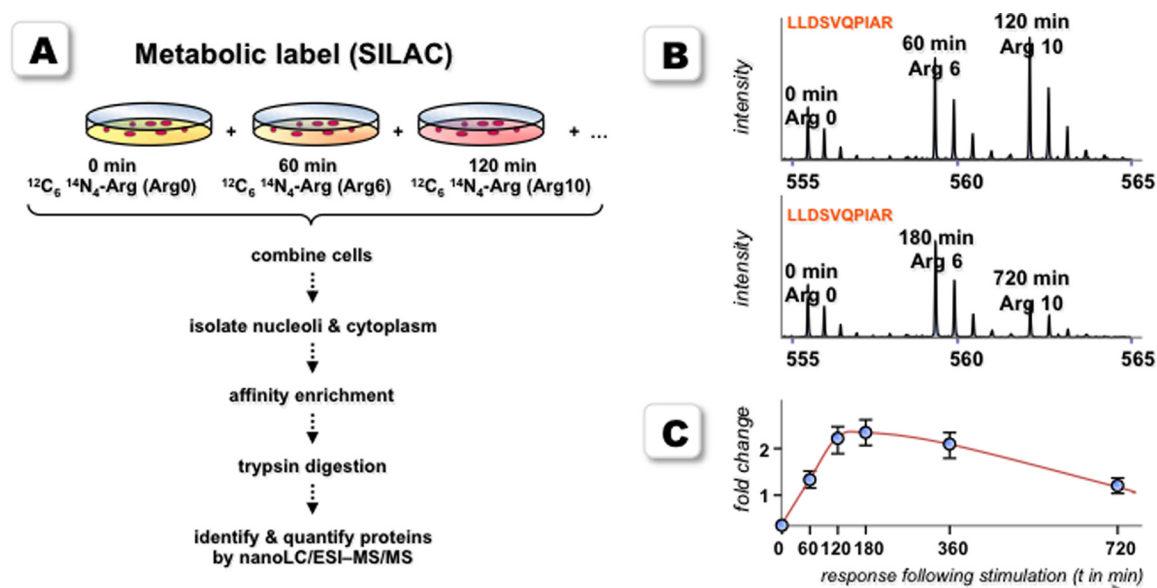


FIG. 2. **Dynamic changes in protein expression revealed through the use of metabolic labeling using stable isotope.** A, cells are first labeled with amino acid isotopes and subsequently biologically stimulated. B, cells are lysed at various time points following stimulation and harvested, and proteins are purified, and peptides are generated. Subsequently, tryptic peptide precursor ion measurements are recorded by MS. C, following normalization and plotting, specific protein expression changes are recorded as a function of time following stimulation.

applicable to study dynamic changes in the composition of protein complexes and their phosphorylation patterns in a site-specific manner in response to cellular stimuli. Their findings led to the identification of dynamic associations between Rad53 and the nuclear transport machinery, histones, and chromatin assembly proteins in response to DNA damage. Interestingly over 30 phosphorylation sites of Rad53 and its associated proteins were identified and quantified, and they observed different changes in phosphorylation in response to DNA damage. More recently Pflieger *et al.* (93) have taken this method one step further to identify *bona fide* proteins that bind to Chico, the insulin receptor substrate, in *Drosophila melanogaster* and insulin-induced changes in the composition of the Chico complex and the state of phosphorylation stoichiometry of these proteins. Following TAP on bait and mock proteins, the samples were first digested with trypsin after which both samples were further divided into halves. The resulting four fractions were subsequently subjected to four-way iTRAQ labeling. In the next step, two of the four iTRAQ-labeled samples were dephosphorylated using a phosphatase and were finally recombined and analyzed by LC-MS/MS. Subsequent data analysis enables one to distinguish true complex components from nonspecific binding proteins in a time-dependent manner to measure changes in the complex composition, reveal the phosphorylation sites, and provide an estimation of the stoichiometry of phosphorylation. Results from this study revealed several new insights to Chico-14-3-3 protein interactions and insulin-dependent stoichiometric changes in phosphorylation on Chico3 and Chico4.

In an earlier study investigating global dynamics of phosphotyrosine-based signaling events in early growth factor

stimulation, Blagoev *et al.* (90) developed a mass spectrometric method based on peptide isotopic abundances to measure molecular changes over time. SILAC reagents were chosen for this analysis (94). The SILAC method is schematically represented in Fig. 2, where one cell population is grown in medium containing normal Arg and another population is grown in ("heavy") medium with ^{13}C -substituted Arg (e.g. $^{12}\text{C}_6$, $^{14}\text{N}_4$ Arg and $^{13}\text{C}_6$, $^{14}\text{N}_4$ Arg) until the Arg is completely incorporated into each protein in the cell. The resulting difference between the two proteomes is only in the Arg-containing proteins, thus rendering their peptides distinguishable by mass spectrometry. Direct experimental errors by humans do not usually occur at the labeling step as the proteome is labeled prior to further experimental steps, and protein synthesis or degradation have no effect on the actual measured signal intensities. To allow simultaneous quantification of three cellular states, the authors introduced a second isotopic label ($^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg), allowing different time points of growth factor treatment (Fig. 2). Following combination of two sets of experiments, they could generate five-point dynamic profiles, allowing identification of 81 signaling proteins, including many EGFR substrates, 31 novel effectors, and their temporal activation upon EGF stimulation. Although the data in this study provide insight into early EGFR receptor signaling, the work also revealed many proteins, as yet unknown, to be linked with EGFR signaling, as well as a variety of EGFR-related phenomena.

Summary—It is well established that PPIs are critical for all cellular functions and that in cellular signaling sophisticated PPI mechanisms are used to define specificity of cellular responses. As signaling protein interactions are often dy-

dynamic in nature and subject to regulation, it is clear that several parallel and overlapping approaches are required to comprehensively map signaling PPIs in any particular cellular system or condition. As an example, a recent study elegantly demonstrated the usefulness of Y2H (95) for the successful identification of substrates for E3 ubiquitin ligases, something an MS-coupled affinity purification could most likely not do. However, the classical Y2H method in all practical cases simply detects direct interactions between two proteins, and it is well established that indirect interactions, typically identified by the MS-coupled protein complex purification methods, can be very important for the core function of the bait protein (56).

At a systematic level, many signaling studies in the past have been described through models that are often only partially mapped, resulting in poorly defined models, gaps in relationships between the communicating and interacting species, and an absence of *in vivo* rate constants. A combination of studying the appropriate methodologies described above should help modeling efforts as follows: 1) providing more complete lists of the molecular makeup of proteins, their PTMs, interactions, and networks; 2) providing information about the recruitment and ratios upon activation (architecture of a complex); 3) establishing the flow of information, through temporal ordering of the molecular phenomena in signaling networks; and 4) establishing rate constraints for models and the components of models, based on the measured activation curves for single proteins.

Such dense collections of data on selected proteins and networks should reveal information about a rich variety of specific bait protein-related phenomena and guide the discovery of new modes of operation in cell signaling. However, because of ever-increasing complexity of PPI data, the actual use of the gathered information will be extremely challenging for researchers without concomitant bioinformatics advances. Therefore, the development of PPI databases, such as the recently published PINA (96), collecting publicly available PPI information (regardless of PPI methodology), and providing user-friendly tools for network construction, filtering, analysis, and visualization of the data will be increasingly important to fully exploit the PPI studies for understanding mechanisms of cell signaling.

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